

## Association of interleukin-10 with hepatitis B virus (HBV) mediated disease progression in Indian population

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**Background & objectives:** Interleukin (IL)-10, an anti-inflammatory Th2 cytokine, is one of the key coordinators of the inflammatory responses involved. The present study was designed to evaluate the impact of *IL-10* (-819/-592) genotypes, haplotypes, mRNA and the protein levels with risk for hepatitis B virus (HBV) related hepatocellular carcinoma (HCC) development in India.

**Methods:** A total of 390 subjects (145 controls, 62 inactive HBV-carriers, 64 chronic-active HBV patients, 60 HBV related cirrhotics and 59 HBV- HCC subjects) were enrolled in the study. Allele specific (AS)-PCR, ELISA and RT-PCR methods were used for assessing polymorphism, spontaneous blood levels and the mRNA expression, respectively of IL-10.

**Results:** The study revealed that the CC/TA genotype acted as a risk factor for cirrhosis (OR<sup>a</sup>=2.02; *P*<0.05) and the subsequent HCC development (OR<sup>a</sup>=2.20; *P*<0.05), with controls as reference. However, no significant association was found between the two haplotypes (CC and TA) observed and HCC risk. Moreover, the IL-10 protein and mRNA levels in peripheral blood mono nuclear cells (PBMCs) showed a significant elevation as the disease progressed to cirrhosis. But, no variation was observed in the IL-10 levels in subjects with different *IL-10* genotypes.

**Interpretation & conclusions:** These preliminary results suggest a strong association of *IL10* (-819/-592) with the HBV infection mediated disease progression, from inactive carrier state to malignancy, in Indian population.

**Key words** Cytokines - haplotype - HBV - HCC - IL-10 - polymorphism

Hepatocellular carcinoma (HCC) is one of the most frequently occurring tumours worldwide, with an estimated annual incidence of over 600,000<sup>1</sup>. India is a low incidence zone for HCC with less than 5 cases per 100,000 persons per annum<sup>2</sup>. Three-quarters of all the cases worldwide are attributed to chronic infection by hepatitis B virus (HBV) and hepatitis C virus (HCV).

A strong genetic component determining the outcomes of HBV infection has been established through various studies. Chronic inflammation, mainly mediated by cytokines, orchestrates a tumour-supporting microenvironment. Thus the risk for developing HCC in HBV infected individuals increases with severity of inflammation and fibrosis. Consistent to this, in

our previous study, we observed a significant positive association of interleukin-1 receptor antagonist (*IL-1RN*) 1/2 genotype with HBV-HCC development, among controls and carriers. Besides, its 2/2 genotype acted as a potential risk factor for hepatitis and subsequent cirrhosis development, among the same groups. Moreover, proinflammatory IL-1B levels significantly elevated with the disease progression to HCC, as compared to controls in Indian population<sup>3</sup>.

IL-10, a Th<sub>2</sub> cytokine, is mainly involved in regulation of inflammatory responses. It was originally described as a mouse Th2 cell factor, inhibiting cytokine synthesis by Th1 cells<sup>4</sup>. IL-10 is a pleiotropic cytokine produced by macrophages, T-helper 2 cells, and B lymphocytes (CD5 subset) and can both stimulate and suppress the immune response. IL-10 has been shown to inhibit various immune functions, such as antigen presentation, cytokine production, macrophage activation, and antigen-specific T-cell proliferation. By interfering with the co-stimulatory function of antigen-presenting cells (e.g. downregulation of class II MHC expression of monocytes and co-stimulatory molecule expression of macrophages), IL-10 reduces antigen-specific T-cell proliferation. IL-10 plays a key role in the oncogenic and metastatic ability of neoplasms like cervical cancer and B-cell lymphoma<sup>5,6</sup>. In the upstream promoter region of the *IL10* gene, the two linked biallelic single-nucleotide polymorphisms (SNPs) at the -819(C/T) and -592(C/A) positions<sup>7</sup>, have been extensively studied. In a study conducted in Japanese population<sup>8</sup> the (-819C/-592C) CC haplotype (33%) was observed to be predominantly present in HBV carriers with HCC against the controls. In Taiwanese and Koreans, the *IL-10* (-592C/C) genotype was found to be associated with an increased risk of HCC<sup>9,10</sup>. IL-10 levels have been shown to be significantly elevated in patients with type B and C chronic liver disease<sup>11,12</sup>. However, little is reported regarding the association of *IL-10* (-819/-592) with hepatitis B disease progression from chronic state to malignancy. Thus, the present study was designed to correlate the *IL-10* (-819/-592) polymorphism, blood IL-10 and the mRNA levels with HCC risk in patients with HBV infection.

### Material & Methods

A total of 390 subjects were enrolled in this study. Group 1 included 145 healthy subjects showing no clinical signs and symptoms to suggest any form of disease and underwent HBsAg screening, so were designated as the control group. These were the relatives or the attendants of the patients attending the Hepatology

OPD at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India during 2007-2012. Group 2 comprised 62 inactive HBV-carriers, HBsAg positive for a period of 6 months or more but with normal levels of transaminases, were HBeAg negative or anti-HBe positive, had serum HBV DNA less than 10<sup>5</sup> copies/ml and no clinical symptoms of liver disease along with no radiological evidence of cirrhosis or varices on endoscopy. Group 3 consisted of 64 chronic-active HBV patients characterized by HBsAg positivity for a period of 6 months or more along with raised transaminases >1.5 × upper limit of normal (ULN), had serum HBV DNA more than 10<sup>5</sup> copies/ml and imaging techniques showing no evidence of cirrhosis or no varices on endoscopy. Group 4 included 60 HBV related cirrhotics, HBsAg positive for a period of 6 months or more along with raised transaminases > 1.5 × ULN, had serum HBV DNA more than 10<sup>5</sup> copies/ml and imaging techniques showing evidence of cirrhosis or varices on endoscopy and ascites detectable by ultrasound. Group 5 included 59 HBV-HCC subjects characterized by concomitant finding of two imaging techniques showing nodule larger than 2 cm with arterial hypervascularization or by one positive imaging technique showing hypervascularization associated with  $\alpha$ -foetoprotein levels higher than 400 ng/ml<sup>13</sup>. Patients attending the Hepatology OPD at Postgraduate Institute of Medical Education and Research, Chandigarh, India, were enrolled consecutively in groups 2, 3, 4 and 5. Based on the genotype distribution data, power of the study was found to be 95 per cent. The protocol of the study was approved by the Institute's Ethics Committee. Informed written consent was obtained from all the subjects. Venous blood (6 ml) was collected from all the subjects. The characteristics of the study are summarized in Table I.

**DNA isolation:** High molecular weight genomic DNA was isolated from the peripheral blood samples using the standard sodium perchlorate method as described by Daly *et al*<sup>14</sup> and was suspended in TE buffer (pH 8.0; 10 mM Tris and 1 mM EDTA) and finally used for *IL-10* genotyping.

**Genotyping of *IL-10* (-819C>T/-592C>A):** The *IL-10* (-819/-592) polymorphism was determined by allele-specific PCR (AS-PCR) based method<sup>15</sup>. The primers used were as follows: Generic primer (antisense): 5'-AGGATGTGTTCCAGGCTCCT-3', PrimerC (sense): 5'-CCCTTGACAGGTGATGTAAC-3' PrimerT (sense): 5'-ACCCTTGACAGGTGATGTAAT-3';  $\beta$ -actin (forward: 5'-AAACTGGAACGGTGAAGGTG-3'

**Table I.** Characteristics of the study subjects

Parameter	Control (Group 1)	Inactive HBV-carrier (Group 2)	Chronic-active HBV (Group 3)	HBV-cirrhotic (Group 4)	HBV-HCC (Group 5)
No. of subjects	145	62	64	60	59
Age (yr) (mean±SD)	35.5±12.77	31.03±10.47**	32.52±13.77*	46±14.14***^	55.31±12.67***#
Median age (yr)	32	28	32	45	55
Male: Female ratio	3.26:1	3.77:1	3.57:1	9:1	18.66:1

*P*\*<0.05 and \*\*\*<0.001, compared to controls (group 1)  
 \**P*<0.001 compared to group 2; ^*P*<0.001 compared to groups 3; #*P*<0.001 compared to group 4

and reverse: 5'-TCAAGTTGGGGGACAAAAAG-3' (Sigma-Aldrich Pvt. Ltd., Bangalore, India). Briefly, the PCR reactions were carried out in 8µl volume consisting of 50 ng genomic DNA, 1xPCR buffer (50 mM KCl, 20 mM Tris HCl, pH 8.4), 0.6U Taq DNA polymerase (MBI Fermentas, Life Sciences, USA), 2.5 µM each sequence specific primer, 1.5 µM each internal control (*β-actin*) primer, 2.5 mM MgCl<sub>2</sub> and 200 µM each dNTP per tube. The PCR conditions were as follows: 95°C for 2 min; 15 cycles of 95°C for 15 sec, 61°C for 1 min, 72°C 40 sec; 95°C for 20 sec, 56°C for 50 sec, 72°C for 50 sec for 25 cycles, with a final extension of 10 min in the last cycle. PCR amplification resulted in a 233 bp (*IL-10*) and 350 bp (*β-actin*) products, when visualized on 2 per cent agarose gel.

**Haplotype and LD (Linkage disequilibrium) analysis:** The construction and analysis of haplotypes of each individual were inferred using the PHASE algorithm developed by Stephens *et al*<sup>16</sup>, which uses a Bayesian approach incorporating *a priori* expectations of haplotypic structure based on population genetics and coalescence theory. Haploview version 2.0.2 (Whitehead Institute for Biomedical Research, USA) was used to estimate LD. The linkage disequilibrium (LD) was expressed as Lewontin's D (ID'I) and the LD coefficient r<sup>2</sup>, between all pairs of biallelic loci.

**Measurement of blood IL-10 levels:** Peripheral venous blood was collected in sterile heparinized tubes and peripheral blood mononuclear cells (PBMCs) were isolated by using the standard Histopaque (Sigma, St Louis, MO, USA) density gradient centrifugation method<sup>17</sup>. Their viability was assessed by trypan dye exclusion test<sup>18</sup> and were later stored at -80°C. For this purpose, PBMCs were lysed in PBS through repeated freeze-thaw cycles before the determination of the IL-10 levels (10<sup>5</sup>cells/well) using enzyme-linked

immunosorbent assay kit (BD OptEIA™ Set Human IL-10, BD Biosciences Pharmingen, CA, USA). Sensitivity of the kit was 7.8 pg/ml. Initially, we tried to measure the circulating (serum) levels, but in majority of the samples, the IL-10 levels were found to be undetectable, which could be probably due to storage or other technical problems. Since we were interested in analyzing the comparative basal levels of IL-10 in various categories of patients, so we determined the levels in stored PBMCs. For that PBMCs were lysed and IL-10 levels were determined as done by Aceti *et al*<sup>19</sup>, who had studied the expression of p-STAT1 in cell lysates of chronic hepatitis C patients.

**Reverse transcriptase (RT)-PCR analysis:** Total RNA from all subjects was isolated from PBMCs (2 x 10<sup>5</sup>cells) by using HiPurA™ Total RNA Miniprep Purification Spin kit (Hi-Media Laboratories Pvt. Ltd., Mumbai, India) and cDNA synthesis was carried out from the purified and intact total RNA (100 µg) according to manufacturer's instructions (RevertAid™ First Strand cDNA Synthesis Kit, MBI Fermentas, Life Sciences, USA). Primarily, the quality of RNA was checked on 1.5 per cent formaldehyde agarose gel electrophoresis. Analysis of RNA revealed the 28S and 18S RNA species and depicted the RNA to be in intact form. Expression of *IL-10* was evaluated using sequence specific primers<sup>20</sup>. PCR was carried out in 10 µl reaction mixture consisting of 1x PCR buffer (50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 20 mM Tris HCl, pH 8.4), 0.6U of Taq polymerase (MBI Fermentas, Life Sciences, USA), 1 µM of primers (Sigma-Aldrich Pvt. Ltd., Bangalore, India) along with 200 µM of each dNTP. In optimized PCR, the initial denaturation step was carried out for 2 min at 95°C. The denaturation, annealing and elongation steps were carried out respectively for 45 sec at 94°C, 45 sec at 65°C (*IL-10*) or 60°C (*β-actin*) and 45 sec @72°C for 40 cycles. The final extension step was carried out

for 10 min @72°C. The primers were designed using Primer3 Input (version 0.4.0) (<http://primer3.ut.ee>). The sequences of the primers used were: *IL-10* (5' to 3')- Forward: TGGTCAAACCCCGTCTCT, Reverse: CTGGAGTACAGGGGCATG;  $\beta$ -*actin* (5' to 3')- Forward: CACTCTTCCAGCCTTCCTT, Reverse: CGGACTCGTCATACTCCT.

The expected PCR products of size 155 and 318 bp were obtained for *IL-10* and  $\beta$ -*actin* (internal control), respectively when electrophoresed on 2 per cent agarose gel. The densitometric analyses of products was determined by using 'Scion image' software ([scion-image.software.informer.com](http://scion-image.software.informer.com)).

**Statistical analysis:** Genotype frequencies were calculated as the number of participants with a particular genotype divided by the total number of participants. Hardy-Weinberg equilibrium was also tested. The statistical significance was depicted by using Pearson *P* value. Association between genotypes/ haplotypes with HCC risk was calculated as odds ratios (ORs) with 95 per cent confidence intervals (CIs), and their statistical significance was determined by chi-square test using SPSS (v.15 for Windows) and PHASE v2.1 softwares, respectively. OR analysis indicates the risk association of a factor (OR>1: positive association/risk factor; OR<1: negative association/protective factor; OR=1: no association). Further, the adjustment of the OR was done by logistic regression analysis. The densitometric analysis was done by SCION Image Software. The non-parametric Kruskal-Wallis one way-

ANOVA and Student's t-test were used to determine the significance of differences in continuous variables using GraphPad Prism (version 5) software ([graphpad-prism.software.informer.com](http://graphpad-prism.software.informer.com)). Multiple comparisons were done by Mann-Whitney test. Wherever, ANOVA was applied, there multiple comparisons were done by Bonferroni test (*P* values adjusted). Pearson's correlation analysis was used to estimate the correlations between different parameters. The association of independent variables with cytokine concentration was assessed by multivariate linear regression analysis using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, version 15.0 for Windows).

## Results

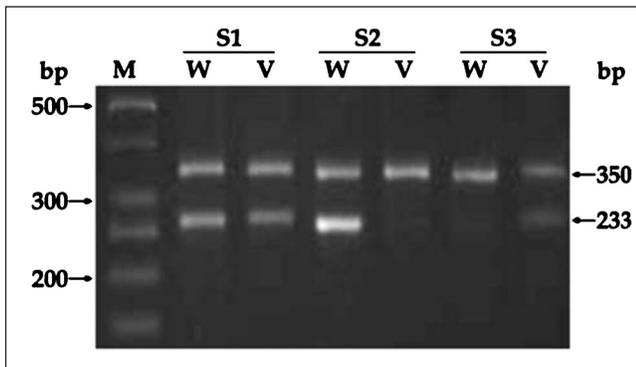
The subjects diagnosed as HBV - HCC showed the highest mean age (55.31 yr) with respect to the other categories. An increased gender ratio (male: female) revealed a distinct male preponderance for HBV - HCC in the present study in all the mentioned groups. The liver function test (LFT) profile depicted that nearly all the investigated parameters were significantly elevated as the disease progresses to cirrhosis, with reference to the controls, thus reflecting an enhancement in degree of disease severity (Table II).

**Interleukin-10 (-819C>T/-592C>A) polymorphism analysis:** As evident from Fig. 1, the presence of *IL-10* (233 bp) band in both the lanes corresponding to wild and variant alleles (sample 1), showed the presence of the heterozygous genotype (CC/TA), while 233 bp

**Table II.** Liver function tests (LFTs) of the different subject groups

Parameter	Control (Group 1)	Inactive HBV-carrier (Group 2)	Chronic-active HBV (Group 3)	HBV-cirrhotic (Group 4)	HBV-HCC (Group 5)	<i>P</i> value (ANOVA)
Total bilirubin (mg/dl)	0.36±0.02	1.14±0.34**	1.08±0.24*	1.57±0.22***	1.12±0.2	<0.001
Total protein (g/dl)	7.18±0.07	7.15±0.17	7.3±0.20	7±0.18	7±0.4	NS
A/G	2.07±0.07	1.78±0.26	1.42±0.21*	1.2±0.1***	1.15±0.21*	<0.001
AST (IU/l)	15.6±0.77	50.36±6.52	78.25±11.64*	129±22.63****	86.43±11.65	<0.001
ALT (IU/l)	12.7±1	50.16±5.56	152.2±79.83**	102.44±24.51**	54.71±7.24	<0.001
ALP (IU/l)	143±3.75	206±19***	281±51***	291±25.4****	260±42***	<0.001

Data expressed as mean ± standard errors (SE); NS, non-significant; AST and ALT, aspartate and alanine aminotransferase, IU, international units; ALP, alkaline phosphatase; A/G, albumin/globulin ratio  
Normal range: Total bilirubin- 0.3-1.3 mg/dl, Total protein- 6.4-8.3 g/dl, A/G- 1.5-2.5, AST and ALT- 2-40 IU/l, ALP- 98-306 IU/l  
*P*\*<0.05, \*\*<0.01, \*\*\*0.001 compared to controls (group 1); *P* +<0.05, +<0.01, +++<0.001 compared to group 2



**Fig. 1.** Allele specific (AS)-PCR analysis of *IL-10* (-819/-592) in some representative samples (S1-S3). Lane M: 50 bp DNA ladder; 350 bp band in all lanes: Internal control ( $\beta$ -actin): S1: CC/TA genotype; S2: CC/CC genotype; S3: TA/TA genotype. S: sample, W: wild allele and V: variant allele.

band was present corresponding to wild allele (sample 2), depicting wild genotype (CC/CC) when the product was amplified against the variant allele alone (sample 3), that showed the variant genotype (TA/TA).

**Genotype distribution of *IL-10* (-819C>T/-592C>A):** The genotype distribution analysis of the polymorphic forms of the *IL-10* (-819/-592) (Table III) showed that compared with the other categories, the wild genotype (CC/CC) was found to be predominant in the control population (24.83%). However, the CC/TA genotype was observed to be maximally present in the hepatitis category (93.75%). Further, the variant genotype (TA/TA) frequency of 15.17 per cent as observed in controls, was significantly higher, as compared to the hepatitis (4.69%;  $P<0.05$ ) and the HCC (5.10%;  $P<0.05$ ) categories. The findings were consistent with the Hardy-Weinberg rule.

**Association of *IL-10* (-819C>T/-592C>A) genotypes with HBV- HCC risk:** The odd's ratio for the association between *IL-10* (-819/-592) genotypes and the carcinoma

progression taking wild genotype as reference (Table IV) showed that the heterozygous genotype (CC/TA) was significantly in positive association or a risk factor for the development of inactive HBV carrier state (unadjusted odds ratio,  $OR^a=4.34$ ;  $P<0.001$ ), cirrhosis ( $OR^a=2.02$ ;  $P<0.05$ ) and subsequently the HCC ( $OR^a=2.20$ ;  $P<0.05$ ), with control population as reference.

**Haplotype distribution of *IL-10* (-819C>T/-592C>A):** The distribution analysis of *IL-10* (-819/-592) haplotypes (Table V) revealed the presence of only two possible haplotypic combinations (wild CC and variant TA). Further, it was observed that all wild type haplotype (CC) was predominantly present in the HBV-cirrhotics (57.50%), while was found to be least in the inactive HBV-carrier category (49.20%). On the contrary, the variant haplotype (TT) was observed to be comparable in carriers (50.80%) and the hepatitis group (50%). Complete linkage disequilibrium ( $D'=1$ ,  $r^2=1$ ;  $P<0.007$ ) was observed in the control group. In contrast, LD was found to be insignificant in the other categories. No significant association of TA haplotype with HCC risk during progression of the disease was observed, among the controls and carriers, with the wild (CC) haplotype as reference (data not shown).

**Analysis of blood *IL-10* levels:** On assaying the spontaneous/unstimulated levels of *IL-10* in the PBMCs (Fig. 2), the levels were found to be increased with the disease progression to cirrhosis. The median level of *IL-10* was not significantly different in the HBV-carrier group (96.2 pg/ml), and the chronic-active HBV subjects (90.76 pg/ml), as compared to controls (78.31 pg/ml). However, the levels were significantly elevated (median: 102.66 pg/ml,  $P<0.01$ ) in the HBV-cirrhotics and were significantly lower in HCC (85.27 pg/ml  $P<0.05$ ). There was a non-significant elevation in *IL-10* (median  $\pm$  SEM) levels, in individuals with CC/TA genotype (80.52  $\pm$  10.71)

**Table III.** Genotype distribution of *IL-10* (-819/-592) among different categories of subjects

Genotype	Control (n=145) (Group 1)	Inactive HBV-carrier (n=62) (Group 2)	Chronic-active HBV (n= 64) (Group 3)	HBV-cirrhotic (n=60) (Group 4)	HBV-HCC (n= 59) (Group 5)
CC/CC	36 (24.83)	5 (8)**	1 (1.56)	10 (16.70)	9 (15.20)
CC/TA	87 (60)	52 (84)***	60 (93.75)***	49 (81.70)**^	47 (79.70)***
TA/TA	22 (15.17)	5 (8)	3 (4.69)*	1 (1.60)	3 (5.10)*

Data represented as number of subjects (genotype distribution, %)  
 $P^* < 0.05$ ,  $^{**} < 0.01$ ,  $^{***} < 0.001$  compared to group 1;  $^{\wedge} < 0.05$ , compared to group 3

**Table IV.** Association of *IL-10* (-819/-592) genotypes with HBV- HCC risk

CC/TA								
Control (n=145) (Group 1)	Inactive HBV-carrier (n=62) (Group 2)		Chronic-active HBV (n= 64) (Group 3)		HBV-cirrhotic (n=60) (Group 4)		HBV-HCC (n= 59) (Group 5)	
1(REF)	OR <sup>a</sup>	OR <sup>b</sup>	OR <sup>a</sup>	OR <sup>b</sup>	OR <sup>a</sup>	OR <sup>b</sup>	OR <sup>a</sup>	OR <sup>b</sup>
	4.34** (1.83-10.3)	4.65* (1.1-19)	ND	-	2.02* (1-4.1)	1.83 (0.6-5.5)	2.20* (1.05-4.5)	1.64 (0.4-6.9)
-	1(REF)		ND	-	0.46 (0.2-1.1)	0.44 (0.1-2)	0.50 (0.2-1.2)	0.41 (0.07-2.5)

Data represented as Odd's ratio (95% confidence interval); ND, not determined due to a single subject having this genotype  
OR<sup>a</sup> = unadjusted odd's ratio and OR<sup>b</sup> = odd's ratio adjusted with age, sex, bilirubin, total protein, A/G, AST, ALT, ALP  
\**P*<0.05; \*\**P*<0.001 compared to control (group 1)

**Table V.** Haplotypic distribution of *IL-10* (-819/-592) among different categories of subjects

Haplotype	Control (n=290) (Group 1)	Inactive HBV-carrier (n=124) (Group 2)	Chronic-active HBV (n= 128) (Group 3)	HBV-cirrhotic (n=120) (Group 4)	HBV-HCC (n= 118) (Group 5)
CC	159 (54.80)	61 (49.20)	64 (50)	69 (57.50)	62 (52.50)
TA	131 (45.20)	63 (50.80)	64 (50)	51 (42.50)	56 (47.50)

Data represented as number of chromosomes examined (haplotype distribution %)

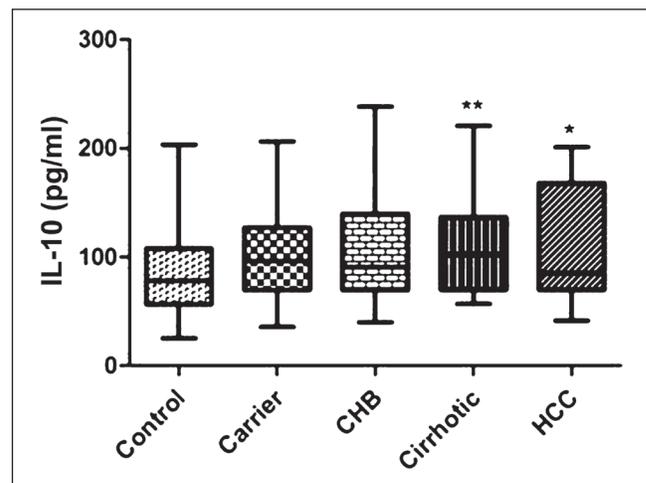
pg/ml when compared to the subjects with CC/CC and the TA/TA genotypes, in case of HBV-carriers (Fig. 3). The Pearson correlation analysis of IL-10, showed a positive correlation between IL-10 levels and the genotypes ( $r = 0.157$ ;  $P < 0.05$ ). Further, on multiple regression analysis (variables assessed in this analysis included *IL-10* genotype, total protein, A/G, OT, PT, billirubin, and alkaline phosphatase), genotypes were also found to significantly affect IL-10 levels.

**RT-PCR analysis of *IL-10*:** The mRNA expression (normalized with  $\beta$ -actin) of IL-10 revealed that the maximum relative expression was observed in cirrhotics, which was 3.4-fold higher than controls ( $P < 0.001$ ), 4-fold higher than carriers ( $P < 0.001$ ) and almost 2-fold more than that in hepatitis group ( $P < 0.01$ ). However, a significant decrease (3.7-fold;  $P < 0.001$ ) was evident, once the disease progressed to HCC (data not shown).

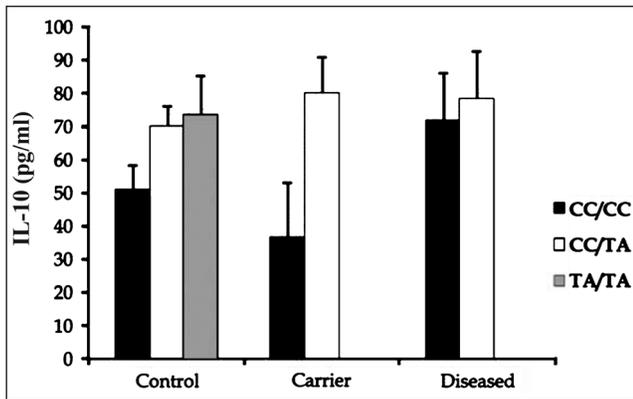
### Discussion

Hepatitis B virus is an infectious agent, causing chronic infection, leading infected patients to various clinical conditions from asymptomatic status to liver cirrhosis, and sometimes to hepatocellular carcinoma (HCC). Since HBV is a noncytolytic virus, the most

part of the pathology in HBV infection is thought to be mediated by the host immune response against the viral products<sup>21</sup>. A shift of Th1/cell-mediated immune responses (involved in viral clearance) to Th2/humoral immune responses (involved in viral persistence) has been implicated in determining the rate of HBV



**Fig. 2.** ELISA analysis of IL-10 levels of various categories. Box and whisker plot depicting median value (middle line) and whiskers showing minimum to maximum values.  $P < 0.05$ , \*\* $P < 0.01$  compared to controls.



**Fig. 3.** IL-10 levels in control, carrier and the diseased groups with different *IL-10* (-819C>T/-592C>A) genotypes. Data represented as (median  $\pm$  SEM) pg/ml. The levels of TA/TA genotype individuals in “carriers” and “diseased” groups, were found to be undetectable.

infection chronicity and HBV-induced liver injury. Moreover, the major Th2/anti-inflammatory cytokine includes IL-10, a potent immuno-modulatory molecule, which is produced by lymphoid cells and exerts its functions by inhibiting macrophage/monocyte and T-cell lymphocyte replication and secretion of proinflammatory cytokines (IL-1, TNF- $\alpha$ , TGF- $\beta$ , IL-6, IL-8 and IL-12)<sup>15</sup>. Chronic liver disease provokes both inflammatory and anti-inflammatory responses, responsible for high IL-10 levels. Active inflammatory status may result in tumour appearance. Keeping this in mind, this study was planned to evaluate the association between the *IL-10* (-819/-592), genotypes, haplotypes, the spontaneous blood levels and the mRNA levels of this cytokine during the progression of hepatitis B infection from the inactive carrier state to the malignancy in the north Indian population. The heterozygous genotype (CC/TA) was the predominant form in both the control and the diseased categories. This observation was found to be consistent with the study done by Roh *et al*<sup>22</sup> in which the same genotype was highest in both controls and cervical cancer patients. Upon studying the association of the genotypes with HBV progression and subsequent HCC risk, the CC/TA genotype was observed to be a significant risk factor and the subsequent HCC development, with controls as reference. On the contrary, no significant association was observed for the same, with HBV-carriers as the referent group. Parallel to this, a study conducted by Nieters *et al*<sup>23</sup> in Chinese population also showed no significant association of wild and the heterozygous genotypes with HCC risk. The haplotype analysis revealed that

the studied single nucleotide polymorphisms (SNPs) were in strong linkage disequilibrium with each other, consistent to the other studies<sup>7,10,15,24</sup>. However, no significant association was found between the two haplotypic combinations (CC and TA) observed and the HCC risk. Similar results were obtained in a study done in the Korean women<sup>22</sup>, where no association between *IL-10* promoter haplotypes (-1082/-819/-592) and cervical cancer risk was observed.

IL-10 blood levels became higher in accordance with the disease progression to cirrhosis. These observations were similar to the earlier studies where elevated serum IL-10 levels have been reported in patients with chronic hepatitis and cirrhosis<sup>25</sup>. Increased levels of IL-10 might reflect the degree of inflammation in the liver<sup>26,27</sup> and increased production of IL-10 might be contributed by many cell types such as CD4<sup>+</sup> T cells, monocytes and macrophages. The levels of IL-10 were found to be low in the HCC patients. Our findings were consistent with a similar study done in the Japanese population<sup>21</sup>. It has been reported that the inter-individual differences in cytokine production appear to be related to allelic polymorphisms of cytokine genes<sup>28</sup>. The cytokine levels are also influenced by various factors, such as inflammation, fibrosis, viral load, viral antigen load, and the occurrence of malignancy in the liver<sup>29-31</sup> and such factors relating to the changes of individual cytokine levels differed with the disease phases. It has been reported that when the patients with HCC are put in immuno-suppressive conditions, the cancer cells themselves may contribute to the conditions by producing immuno-suppressive agents, such as transforming growth factor beta 1 (TGF- $\beta$ )<sup>32</sup>. Numerous other studies have also reported the elevated serum levels of IL-10 in HBV patients compared with controls<sup>28,33,34</sup>. On the contrary, in a study conducted by Song *et al*<sup>11</sup> in HBV-infected Vietnamese patients, the IL-10 levels were found to be a poor predictor of the disease outcome with no association with the clinical presentation of HBV. These results are in contradiction to the present study. The difference in results can be explained on the basis, that Song *et al*<sup>11</sup>, have determined the circulating IL-10 levels, while in our study PBMCs were used for analysis. No significant differences were observed, on correlating the genotypes with the blood levels of IL-10, consistent with the findings of Roh *et al*<sup>22</sup>. Further, there was increased IL-10 expression in the HBV and the cirrhotic groups, with reference to controls. A similar elevated mRNA levels were reported in the diseased group in a study done in HIV-HCV

co-infected Chinese population<sup>35</sup>. In the present study, the mRNA and the protein levels of IL-10 were found to be consistent with each other indicating the increased synthesis of IL-10 in the diseased groups.

The major limitation of the study was the small sample size, which demands a further validation of the findings obtained, by carrying out studies on a larger set of population. Also, HBV genotyping and core promoter mutations were not assessed in this study, so that could be a confounder to the findings presented.

In conclusion, a positive association of *IL-10* (-819/-592) CC/TA genotype with HBV related disease progression and risk of HCC development was observed. Based on the protein and mRNA levels analysis, the present study indicates that IL-10 is a potential candidate gene, strongly associated in predicting the transition of HBV related disease phases.

### References

- But DY, Lai CL, Yuen MF. Natural history of hepatitis-related hepatocellular carcinoma. *World J Gastroenterol* 2008; 14 : 1652-6.
- El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 2012; 142 : 1264-73.
- Saxena R, Chawla YK, Verma I, Kaur J. Interleukin-1 polymorphism and expression in hepatitis B virus-mediated disease outcome in India. *J Interferon Cytokine Res* 2013; 33 : 80-9.
- Redpath S, Ghazal P, Gascoigne NR. Hijacking and exploitation of IL-10 by intracellular pathogens. *Trends Microbiol* 2001; 9 : 86-92.
- Holland G, Zlotnik A. Interleukin-10 and cancer. *Cancer Invest* 1993; 11 : 751-8.
- Nabioullin R, Sone S, Mizuno K, Yano S, Nishioka Y, Haku T. Interleukin-10 is a potent inhibitor of tumor cytotoxicity by human monocytes and alveolar macrophages. *J Leukoc Biol* 1994; 55 : 437-42.
- Breen EC, Boscardin WJ, Detels R, Jacobson LP, Smith MW, O'Brien SJ, et al. Non-Hodgkin's B cell lymphoma in persons with acquired immunodeficiency syndrome is associated with increased serum levels of IL-10, or the IL-10 promoter -592 C/C genotype. *Clin Immunol* 2003; 109 : 119-29.
- Migita K, Miyazoe S, Maeda Y, Daikoku M, Abiru S, Ueki T, et al. Cytokine gene polymorphisms in Japanese patients with hepatitis B virus infection-association between TGF-beta1 polymorphisms and hepatocellular carcinoma. *J Hepatol* 2005; 42 : 505-10.
- Shin HD, Winkler C, Stephens JC, Bream J, Young H, Goedert JJ, et al. Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10. *Proc Natl Acad Sci U S A* 2000; 97 : 14467-72.
- Tseng LH, Lin MT, Shau WY, Lin WC, Chang FY, Chien KL, et al. Correlation of interleukin-10 gene haplotype with hepatocellular carcinoma in Taiwan. *Tissue Antigens* 2006; 67 : 127-33.
- Song le H, Binh VQ, Duy DN, Kun JF, Bock TC, Kreamsner PG, et al. Serum cytokine profiles associated with clinical presentation in Vietnamese infected with hepatitis B virus. *J Clin Virol* 2003; 28 : 93-103.
- Falasca K, Ucciferri C, Dalessandro M, Zingariello P, Mancino P, Petrarca C, et al. Cytokine patterns correlate with liver damage in patients with chronic hepatitis B and C. *Ann Clin Lab Sci* 2006; 36 : 144-0.
- Zhou L, Liu J, Luo F. Serum tumor markers for detection of hepatocellular carcinoma. *World J Gastroenterol* 2006; 12 : 1175-81.
- Daly AK, Steen VM, Fairbrother KS, Idle JR. CYP2D6 multiallelism. *Methods Enzymol* 1996; 272 : 199-210.
- Shin HD, Park BL, Kim LH, Jung JH, Kim JY, Yoon JH, et al. Interleukin 10 haplotype associated with increased risk of hepatocellular carcinoma. *Hum Mol Genet* 2003; 12 : 901-6.
- Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* 2001; 68 : 978-89.
- Boyum A. Separation of lymphocytes, granulocytes, and monocytes from human blood using iodinated density gradient media. *Methods Enzymol* 1984; 108 : 88-102.
- Avelar-Freitas BA, Almeida VG, Pinto MC, Mourão FA, Massensini AR, Martins-Filho OA, et al. Trypan blue exclusion assay by flow cytometry. *Braz J Med Biol Res* 2014; 47 : 307-15.
- Aceti A, Zechini B, Griggi T, Marangi M, Pasquazzi C, Quaranta G, et al. Undetectable phospho-STAT1 in peripheral blood mononuclear cells from patients with chronic hepatitis C who do not respond to interferon- $\alpha$  therapy. *Liver Int* 2005; 25 : 987-93.
- Kothari ST, Deshmukh RA. Estimation of mRNA levels of interleukin (IL)-10, tumor necrosis factor (TNF) -  $\alpha$ , IL-4 and interferon (IFN)- $\gamma$  in HIV infected children in Mumbai. *Indian J Clin Biochem* 2006; 21 : 15-26.
- Ayada M, Ishikawa T, Okumura A, Tanabe J, Ito H, Ohashi T, et al. Alteration of serum cytokine balances among different phases of chronic hepatitis B virus infection. *Hepatol Res* 2006; 34 : 214-21.
- Roh JW, Kim MH, Seo SS, Kim SH, Kim JW, Park NH, et al. Interleukin-10 promoter polymorphisms and cervical cancer risk in Korean women. *Cancer Lett* 2002; 184 : 57-63.
- Nieters A, Yuan JM, Sun CL, Zhang ZQ, Stoehlmacher J, Govindarajan S, et al. Effect of cytokine genotypes on the hepatitis B virus-hepatocellular carcinoma association. *Cancer* 2005; 103 : 740-8.
- Gambhir D, Lawrence A, Aggarwal A, Misra R, Mandal SK, Naik S. Association of tumor necrosis factor alpha and IL-10 promoter polymorphisms with rheumatoid arthritis in North Indian population. *Rheumatol Int* 2010; 30 : 1211-7.
- Chau GY, Wu CW, Lui WY, Chang TJ, Kao HL, Wu LH, et al. Serum interleukin-10 but not interleukin-6 is related to clinical outcome in patients with resectable hepatocellular carcinoma. *Ann Surg* 2000; 231 : 552-8.

26. Kakumu S, Okumura A, Ishikawa T, Yano M, Enomoto A, Nishimura H, *et al*. Serum levels of IL-10, IL-15 and soluble tumour necrosis factor-alpha (TNF-alpha) receptors in type C chronic liver disease. *Clin Exp Immunol* 1997; 109 : 458-63.
27. Cacciarelli TV, Martinez OM, Gish RG, Villanueva JC, Krams SM. Immunoregulatory cytokines in chronic hepatitis C virus infection: pre- and posttreatment with interferon alfa. *Hepatology* 1996; 24 : 6-9.
28. D'Alfonso S, Rampi M, Rolando V, Giordano M, Momigliano-Richiardi P. New polymorphisms in the IL-10 promoter region. *Genes Immun* 2000; 1 : 231-3.
29. Bozkaya H, Bozdayi M, Türkyilmaz R, Sarioglu M, Cetinkaya H, Cinar K, *et al*. Circulating IL-2, IL-10 and TNF-alpha in chronic hepatitis B: their relations to HBeAg status and the activity of liver disease. *Hepatogastroenterology* 2000; 47 : 1675-9.
30. Hyodo N, Nakamura I, Imawari M. Hepatitis B core antigen stimulates interleukin-10 secretion by both T cells and monocytes from peripheral blood of patients with chronic hepatitis B virus infection. *Clin Exp Immunol* 2004; 135 : 462-6.
31. Hyodo N, Tajimi M, Ugajin T, Nakamura I, Imawari M. Frequencies of interferon-gamma and interleukin-10 secreting cells in peripheral blood mononuclear cells and liver infiltrating lymphocytes in chronic hepatitis B virus infection. *Hepatol Res* 2003; 27 : 109-16.
32. Okumoto K, Hattori E, Tamura K, Kiso S, Watanabe H, Saito K, *et al*. Possible contribution of circulating transforming growth factor-beta1 to immunity and prognosis in unresectable hepatocellular carcinoma. *Liver Int* 2004; 24 : 21-8.
33. Tulek N, Saglam SK, Saglam M, Turkyilmaz R, Yildiz M. Soluble interleukin-2 receptor and interleukin-10 levels in patients with chronic hepatitis B infection. *Hepatogastroenterology* 2000; 47 : 828-31.
34. Wang J, Ni H, Chen L, Song WQ. Interleukin-10 promoter polymorphisms in patients with hepatitis B virus infection or hepatocellular carcinoma in Chinese Han ethnic population. *Hepatobiliary Pancreat Dis Int* 2006; 5 : 60-4.
35. Gonzalez SA, Zhang C, Fiel MI, Chung S, Zhang L, Jacobson IM, *et al*. Hepatic inflammatory cytokine mRNA expression in hepatitis C virus-human immunodeficiency virus co-infection. *J Viral Hepat* 2008; 15 : 331-8.

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